

Vitamin D status among pre-school children in rural Nepal

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The Project

Title: ‘Vitamin D status among children age group 1-5 years of old in rural Nepal at latitude 27,39⁰ N’

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Preface

Presentation plan

This thesis submitted as a part of the Master of Philosophy Degree in International Community Health. Current thesis structured according to the *Requirements of the MPhil thesis*, February 2013, Department of Community Medicine, option two (one article submitted to an international peer reviewed journal plus a Summary).

Summary of the thesis is started with an abstract of the article submitted for the publication. Thesis includes introduction and methodological consideration with detailed methods and materials. Result and discussion of the findings are not included. Copy of the submitted article included.

Abbreviation

ARTI:	Acute Respiratory Tract Infections
CHV:	Community health volunteers
DBP:	Vitamin D binding protein
DBS:	Dried Blood Spots
ID:	Identification
LC-MS/MS:	Liquid Chromatography - Tandem Mass Spectrometry
LSES:	Low socio-economic status
NR:	Nepali Rupee
NHRC:	Nepal Health Research Council
PTH:	Parathyroid Hormone
RSV:	Respiratory Syncytial Virus
TLR:	Toll-like receptors
UVB:	Ultraviolet B
USES:	Upper socio-economic status
VDC:	Village Development Committee
VDR:	Vitamin D receptor
VDRE:	Vitamin D response element
WHO:	World Health Organization
7DHC:	7-dehydrocholecalceferol
25(OH)D:	25-hydroxyvitamin D
1,25(OH) ₂ D:	1,25-dihydroxyvitamin D

Abstract

Vitamin D status among pre-school children in rural Nepal determined by using dried blood spot sampling.

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Insufficiency of vitamin D, which plays a major role in the calcium and bone metabolism, is reported from populations worldwide. Its extra skeletal benefits are being identified, vitamin D is important in function of immune and CV systems. However, little is known about the vitamin D status among populations from developing country settings, particularly in children.

Objective: we aimed to assess the vitamin D status and contributing factors among children between 1 to 5 years of age in rural Nepal at latitude 27.39° N.

Materials and methods: A total of 280 children aged between 1 and 5 years were randomly selected from the records of vitamin A supplementation program and included in the study. Blood samples were collected using the dried blood spot technique. The level of serum 25-hydroxyvitamin D₃ (25(OH)D₃) and D₂ (25(OH)D₂) were measured in whole dried blood spots by using liquid chromatography- tandem mass spectrometry analysis. For the assessment of calcium intake and background variables we used structured questionnaire.

Results: Hypovitaminosis D, defined as a 25(OH)D₃ level less than 50nmol/l was identified in 91.1% of children. There was no association between level of 25(OH)D₃ and gender, socio-economic indicators, sun exposure or nutritional status.

Conclusion: vitamin D deficiency is common among children from 1-5 years of old living in rural Nepal.

Keywords: Vitamin D deficiency, Dried blood spot, Child health, South Asia

1. Chapter I, Introduction

1.1 Literature review

1.1.1 Vitamin D photobiology and physiology

Vitamin D is prohormone that plays an essential role in the mineralisation of bones (1). There are two ways for humans to meet requirement for vitamin D, the major amount produced in the skin after exposure to the sunlight and the rest is fulfilled from dietary sources. There are few foods that are naturally rich with vitamin D, although there are some fortified products available for consumption (2).

Vitamin D exists in two forms, vitamin D₂ (ergocalciferol) generated from ergosterol in plants and vitamin D₃ (cholecalciferol) is produced in the skin of humans as well as by some animals from 7-dehydrocholecalciferol (7DHC), both reactions are prompt due to exposure to sunlight (3).

The process of vitamin D production in human's cutaneous from 7DHC consists of two main stages. In the skin 7DHC under the impact of UVB radiation with the wavelengths 290-315 nm enters into the reaction of isomerisation and generates previtamin D₃. Subsequently, previtamin D₃ undergo a reaction prompt by the skin temperature and with help of membrane fatty acids is transformed into the vitamin D₃. The next step is shifting vitamin D₃ from the skin to the circulation by vitamin D binding protein (DBP). Obviously, it is not possible to develop hypervitaminosis due to excessive insolation, the excess of vitamin D is destroyed by photodegradation in the skin by UVA, in this way mankind maintains homeostasis and provides tissues and organs with essential amount of vitamin D (4).

The further metabolism of vitamin D is happening in the liver and kidneys. Vitamin D that was produced in the skin or taken from diet is processed in the liver by cytochrome P450s to 25-hydroxyvitamin D (25(OH)D) (5).

25(OH) D is main circulated form out of whole pool of vitamin D metabolites and it has half-life of two-three weeks. Therefore for the assessment of vitamin D status the 25(OH) D is most appropriate metabolite to be measured, which reflects well with secondary hyperparathyroidism, rickets and osteoporosis (5). Subsequently, in the kidneys 25(OH) D is metabolised by 1 – alpha hydroxylase to the biological most active form is

1,25dihydroxyvitamin D ($1, 25(\text{OH})_2\text{D}$). All farther effects of vitamin D on the health of humans occurs through the acting of 1,25dihydroxyvitamin D (6).

1.1.2 Effect on the skeletal system

$1, 25 (\text{OH})_2\text{D}$ plays an essential role to sustain the constant level of calcium and phosphorus in the circulation. The key point of the regulation is presence of vitamin D receptors (VDR) in the small intestinal cells and in osteoblasts of skeletal system. By the interaction with VDR, $1, 25 (\text{OH})_2\text{D}$ increases the reabsorption of calcium and phosphorus in the small intestine. In case of insufficient dietary intake of calcium, $1, 25 (\text{OH})_2\text{D}$ interacts with VDR in osteoblasts. This interaction mobilizes the calcium storage from the bones by activating the proliferation process of osteoclasts (6).

In the conditions of vitamin D deficiency, the absorption of calcium and phosphorus in small intestine may decrease up to 85-90% and 40% respectively (5). It leads to declining the level of ionized calcium in serum; the low level of ionized calcium in serum causes irritation of calcium sensors in the parathyroid glandules. In response, parathyroid glandules produces more parathyroid hormone (PTH). As a result of hyper secretion of PTH, the reabsorption of calcium in the kidneys' tubular system is elevated, while phosphorous is loosed with urine. In addition, PTH dissolves bones to maintain normal Calcium level in the serum. Moreover, in the state of secondary hyperparathyroidism the conversion of $25(\text{OH})\text{D}$ into $1, 25(\text{OH})_2\text{D}$ is enhanced. Therefore, it is not informative to measure the levels of $1, 25(\text{OH})_2\text{D}$ or calcium, because its remains normal while person might have deep deficient of vitamin D (6).

Vitamin D deficiency leads to comprehensive failure in the mineralization of the bones and development of osteomalacia for children when skeletal system is immature it manifests in form of rickets and for adults osteoporosis(6) .Additionally, it is known that children with vitamin D deficiency status in early life develop grow retardation, it may have implication for the future life due to inability to achieve genetically inherent height (3).

1.1.3 Extra skeletal effects of vitamin D

During recent years the role of vitamin D for human health is reappraised. The numbers of review articles have been published to summarise the extra skeletal effects of vitamin D (7-12). It has been suggested that the compensate state by vitamin D is not only essential for normal development and function of skeletal system but also may play important role in the prevention of autoimmune, neoplastic and cardiovascular diseases. (7). Moreover, there is evidence that vitamin D deficiency may contribute to the incidence and the severity of acute low respiratory tract infections as well as progression of tuberculosis to its active form (12-14).

Due to discovery that the nuclear VDR presents in the majority of human's tissues (7), and understanding that the most type of cells are able to transform vitamin D into the its active form 1, 25(OH)₂D, the interest to non-skeletal effects of vitamin D appears (15).

1, 25(OH)₂D is recognized as a steroid hormone that acts as a gene expression factors. 1, 25(OH)₂D is attaching to the VDR with association of ligand-activated transcriptional factors and displaces into the nucleus. Inside of nucleus 1, 25(OH)₂D binds to the special DNA sequences recognized as the vitamin D response elements (VDRE) (7). Hereby, vitamin D regulates expression of more than 200 genes with transcription of various proteins. Hence, these proteins adjust cellular differentiation, proliferation and apoptosis with consequent effects on the function of many organs and systems (14, 16).

As a matter of fact, for paediatric group the potential effect of vitamin D on immune system and risk of acquiring respiratory infections is more crucial and influential on public health indicators.

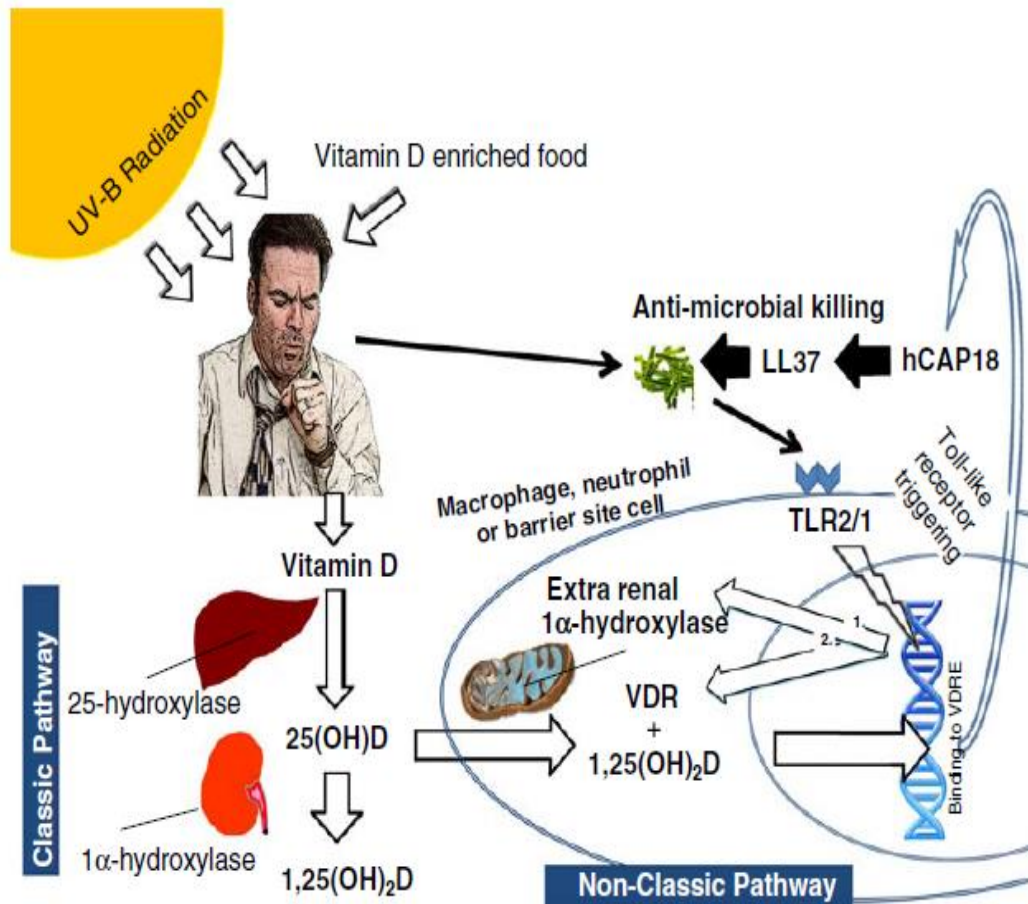


Figure 1: Vitamin D's pathways (16)

1.1.4 Potential effects of vitamin D on the Immune system

As pertaining to immune system, the skin, gastrointestinal tract and respiratory tract serve as an ordinary portal of infections entry. While, as a part of innate immune system epithelial cover together with macrophages and neutrophils provide barriers against infection(17).

Circulated 25(OH)D is absorbed by macrophages, neutrophils and epithelial cells. In the cellular level under the impact of extra renal 1 alpha hydroxylase 25(OH)D converted into the active form 1,25 (OH)₂D. Consequently active vitamin D banded to VDR and after the translocation into the nucleus attaches to the VDRE (16). It is known that gene encoded for cathelicidin accommodate VDRE (18). Cathelicidin is antimicrobial peptides produced by epithelial cells and neutrophils and relates to the function of innate immune systems(15). (See figures1, vitamin D's pathways)

1,25(OH)₂D-VDRE complex turn on the gene responsible for the synthesis of cathelicidin (hCAP18). Cathelicidin undergoes through the segmentation and is transformed into the active form IL₃₇(16). In fact, IL₃₇ manifests antimicrobial activities against bacteria, virus and fungi(15) .

Additionally, on the surface of macrophages there are Toll-like receptors (TLRs) they play role of sensors to recognize bacterial lipoprotein and forward signal to macrophage for synthesis of –cathelicidin. As a part of this signalling system, when Toll-like receptors are triggered by bacteria's lipoprotein it is boost VDR and extra renal 1 alpha hydroxylase, it leads to the enhancement of the cathelicidin's production (16).

The recent immunological data indicates that conversion of vitamin D into the active form 1,25(OH)₂D is permanent process in the epithelial cells of respiratory tract and it accelerates during the viral infection (19). Therefore, sufficient status of vitamin D is essential for the adequate cathelicidin production as a part of defence reaction against respiratory infections (15).

Numbers of epidemiological studies have been conducted that have looked into possible association of vitamin D deficiency and acute low respiratory tract infections among paediatric group (20, 21). Results from case-control studies conducted in India (22), Bangladesh (23) indicated strong association between vitamin D deficiency with acute low respiratory tract infections (ARTI). However, this association has not been proved for Canadian children with bronchiolitis, studied by Roth et al. in 2005 (24); although, the authors suggested that it could be due to different etiological factors that cause ALRI in developing countries and in developed one such as Canada(24). Later, McNally et al. conducted another case- control study in Canada, it has compared vitamin D status between children with pneumonia and healthy ones (25).The result has provided some new concept. It has been suggested that vitamin D deficiency is not associated with incidence of ALRI, but with severity of respiratory infections among paediatric group (25). This conclusion has been confirmed by hospital based retrospective case study from Japan(26). Furthermore, complementary analysis of the Canadian study by Roth et al. from 2005 (24), identified association between genotype *ff* with less active VDR in the epithelial cells of respiratory tract (12). It has been show that children with genotype *ff* susceptible to ARTI particularly towards RSV (respiratory syncytial virus) bronchiolitis, because of inability of vitamin D to implement immunomodulatory and antimicrobial effects (12, 27). Additionally, data from

study conducted in a vitro model suggested that vitamin D highly likely diminishes inflammation in respiratory tract caused by RSV (19), also there is evidence that vitamin D is able to suppress release of pro inflammatory cytokines by macrophages (18).

In fact, interventional randomized control trials were called to resume the vitamin's D effects on the susceptibility towards acute low respiratory tract infections in paediatric group. Several trials have been conducted at the different settings among the population at high risk of vitamin D deficiency (28-30). The results vary from positive effect on the reduction of incidence of repeated cases to no effect of intervention. Nevertheless, it has been suggested that intervention may require adjustment in dosage and regiment or effect from supplementation may be different for the different age groups and further research is needed (31).

As regards to the association of vitamin D status and tuberculosis, the meta-analysis and systematic review published in 2008, concluded that there is a strong correlation between vitamin D insufficiency and risk of development active tuberculosis (13). In addition the result from cohort study indicates, that the low status of vitamin D increases the probability to develop acute tuberculosis by 5 folds among healthy household contacts(32). The reason is that in the low vitamin D status the synthesis of antimicrobial peptide catelicidin by macrophages and respiratory epithelial cells is decreased, hence increase susceptibility towards Mycobacterium Tuberculosis (13, 32, 33).

1.1.5 Determination of vitamin D status

For the assessment of vitamin D status it is recommended to measure 25(OH)D; it has half-life of 2-3 weeks and considered as the most reliable metabolite that reflects the body's vitamin D stores. In contrast as it was discussed above the measurement of its active metabolite 1,25(OH)₂D is not informative, and is recommended only if there is suspicion on impaired production of 1,25(OH)₂D by kidneys in terms of rare inherit or acquired disorders (34).

As regards to the measurements techniques of various methods are available such as radioimmunoassay and high-performance liquid chromatography. However, up to day the most accurate method to quantify 25(OH)D is liquid chromatography – tandem mass

spectrometry (LC-MS/MS), it allows to measure 25(OH)D₂ and 25(OH)D₃ separately and is considered as a gold standard(35).

In addition, during recent years the dried blood spot(DBS) sampling methods along with LC-MS/MS has been suggested as a new approach for the clinical testing and screening(36). This novel method of sampling brings better opportunities for the assessment of vitamin D status. First of all it is less invasive and consequently reduces the risk of infection's transmission; secondly for the assessment the amount of required blood is remarkably less, what makes it convenient for paediatric patients. Another advantage it does not require sophisticated equipment for storing and shipping materials, so can be used for field research in the resource poor setting (36). And the most important, it has shown good correlation between LC-MS/MS method using DBS with serum. As a result LC-MS/MS method using DBS for assessment of vitamin D considered to be high sensitive and correlated good with serum level(37-39).

As regards evaluation of vitamin D status, so far there is not unified agreement for the definition of vitamin D sufficiency and insufficiency. For the determination of cut off level were suggested various methods. The most common way of determining normal level of vitamin D is to identify the minimum level of 25(OH)D which maximum suppress the secretion of PTH, consequently plateau of PTH was observed at level 30ng/ml (34). However, there is some discrepancies, it was identified that for some individuals there is no correlation between level of 25(OH) D and PTH. Moreover, the deviation in the level of PTH does not always related to the changes in the vitamin D status during childhood, because of elevated calcium absorption in the period of active growth (40).

There are also different suggestion regarding the normal range of vitamin D, such as determine the level of 25(OH) D that ensures maximal absorption of calcium in the intestinal, or the level when the majority does not have any manifestation of diseases associated with vitamin D metabolism. However, both of these suggestions are also debateable (40).

In fact, until recently the most widely accepted definition of vitamin D status was the one suggested by Lips P. (41), where vitamin D deficiency subdivided on mild, moderate and sever deficiency.

Table 1: Definition of Vitamin D status

Vitamin D status	Level of 25(OH)D
Severe deficiency	0-12.5 nmol/l
Moderate deficiency	12.6-25 nmol/l
Mild deficiency	25.1-49.9 nmol/l
Sufficient	≥ 50 nmol/l

Although, these criteria still widely accepted by clinicians (see table above), the Endocrine Society's Clinical Practice Guidelines suggested the new criteria for both children and adults (see table below) (42).

Table 2: Definition of vitamin D status by Endocrine Society's Clinical Practice Guidelines

Vitamin D status	Level of 25(OH)D	
	ng/ml	nmol/litre
Deficiency	20	50
Insufficiency	21-29	52.5-72.5
Sufficient	30	75
Ideal	30-60	75-150
Safe	<100	250

It has been suggested, that the level of 25(OH)D 10ng/ml is needed to maintain the bone metabolism (40), although the level of vitamin D that requires to perform extra skeletal effects is greater and more likely is 30ng/ml (18).

Additionally, it is recommended for assessment of child's vitamin D status to measure level of calcium, phosphorus and alkaline phosphatase. Alkaline phosphatase is marker of bone turn over and the level of calcium will be deviated when the bone stores are depleted. So, these indicators are only informative when child is severe deficient and bone metabolism is involved (40).

1.1.6 Risk factors for developing hypovitaminosis D

Geographical determinants, it is known that living above 35°N latitude is heightening the risk of vitamin D deficiency during winter months. The reason is that considerably less UVB photons are able to reach the Earth's surface in that latitude; the UVB photons are absorbed by the ozone layer in the stratosphere when the zenith angle is oblique as it is happening during winter months (3). Moreover, it has been recently proved by Holick et al. (43) in vitro models that not only latitude does matter but also the height above sea level. The altitude has considerable effect on the production of vitamin D from 7DHC. It has been compared the production of vitamin D in three different altitudes in India and Nepal during last week of October to first week of November, Agra 169m above sea level, Kathmandu 1400m, mountain Everest 5350m. The differences were remarkable, it has been concluded that in the latitude 27°N on the area with altitude below 3400m production of vitamin D declined significantly (43). Another factor is pollution; atmospheric pollution may have great impact on the vitamin D status, through enhancement of UV photons absorption. Although it is more essential for inhabitants of big industrialized cities with high level of emission (44).

No less important are skin pigmentation and use of sunscreen. Skin pigmentation is determined by melanin, which is produced in the basal layer of epidermis by melanocytes. Melanin works as a photo protector, it has great capacities to absorb UVB and decrease syntheses of vitamin D (43). As well as the proper use of sunscreen with protection factor 8, consumes up to 95% of UV photons, and respectively reduce the production of vitamin D in the skin (43).

Another determinant is restriction of sunlight exposure it could be due to clothing habits or limitation of outdoor activities. Also, scarcities of products in the diet naturally rich with vitamin D and absence of food fortification policy in the country would have certain impact on the vitamin D status of population (45).

Additionally, exclusively breastfeeding is a predisposing factor for the development of vitamin D deficiency. It is known that breast milk contains not enough vitamin D to provide child with daily requirement, it consists even less when women is vitamin D insufficient. Consequently it is recommended to ensure sun exposure for infants when it is possible, or provide supplementation (1).

Yet other risk factors for the developing of vitamin D insufficiency or deficiency are the conditions that cause malabsorption, hence decrease the intake of vitamin D from intestine. Also, the important role plays the treatment with medication that is likely to interfere with vitamin D metabolism (Phenytoin, Phenobarbital, Carbamazepin, Isoniazid, Theophylline, Rifampin) (1).

During recent years one more determining factor was lifted up. It has been shown that people with obesity at the risk of vitamin D insufficiency due to reduced bioavailability of vitamin D. In other words they develop relative insufficiency because vitamin D deeply sequesters in the fatty tissue and cannot replenish the circulating pool of vitamin D(46).

Each of these factors may contribute to the development of vitamin D deficiency in a certain extent. For South Asian region, skin pigmentation, clothing style, traditional diet and exclusively breastfeeding are the major factors. Additionally for the industrial population it is account pollution and time spend outdoor (45).

1.1.7 Recommendations for vitamin D testing

According to the resent scientific data it is advised to test those who are at risk of development insufficiency or deficiency (1, 40, 42):

- Individuals with darker skin and living at high latitude
- Individuals with chronic diseases that leads to the malabsorption or those who are on the long lasting medication that interferes with vitamin D metabolism
- Infants with symptoms of rickets
- Individuals with frequent fractures

There is not data on the benefits of testing general population (42).

1.1.8 Recommendation for vitamin D supplementation

In the recent past the recommendation for vitamin D supplementation regarding infants was 200 IU/daily, the dosage was calculated based on the evidence that 200 IU/d allows to keep the level of 25(OH)D on the level of 11ng/ml (1). However, in connection with recent knowledge of normal vitamin D status, the recommendation was revised. The most updated recommendations based on the suggestion of maintaining the level of vitamin D equal to

30ng/ml. The Endocrine Society’s Clinical Practice Guidelines suggested the following dosage for the prevention (table 4) and for the treatment (table 5) of vitamin D deficiency (42).

Table 3: Recommendation regarding vitamin D intake for the prevention of deficiency

Group of Individuals	Vitamin D ₃ or Vitamin D ₂
Children 0-12 months	400- 1000 IU*
Children 1-8 years	600-1000 IU
Children (males) 9-18 y	600-1000 IU
Children (females) 9-18 y	400-2000IU
Adults	1500-2000 IU
Pregnancy, and lactation period	1500-2000 IU
Mother’s requirement during exclusively breastfeeding, if child does not take supplementation	2000 - 4000 IU

*1000 IU= 25 mcg

Table 4: Recommendation regarding vitamin D intake for treatment of deficiency

Group of individuals	Vitamin D ₃ or Vitamin D ₂
Children 0-12 months	2000 IU/daily during 6 weeks, after reaching level of 30ng/ml → 400 IU/daily
Children 1-18 year	2000 IU/daily during 6 weeks, after reaching the target level is 30ng/ml→ 600 IU/ daily
Adults	50,000 IU/weekly during 8 weeks, after reaching target level is 30ng/ml→ 1500 IU/daily

Additionally, for obese individuals (BMI 30 kg/m² or more) as well as for those who are on the continuing medication that interferes with vitamin D metabolism it is recommended augment the dosage twice (42).

In fact, the Endocrine Society’s Clinical Practice Guidelines, stated clear that there is no evidence regarding differences in outcomes of vitamin D2 or vitamin D3 supplementation;

and in addition there is not significant differences whether to comply daily, weekly or quarterly regiment (42).

1.1.9 Vitamin D status around the world

During recent years numbers of systematic reviews have highlighted worldwide prevalence of vitamin D deficiency (45, 47, 48). In fact, the authors were faced with difficulties of comparing various studies from different regions. It is known that researcher has been using different assay methods to measure the level of 25(OH) D. Additionally, the level of vitamin D which is qualified as normal or insufficient quite debatable, although most of researcher agree that the level of 25(OH) D should be at least 50 nmol/l to be considered as sufficient. Nevertheless, due to these reviews some general trends are recognizable. It could be concluded that vitamin D deficiency is worldwide health issue, although the contributing factors varies for different regions (48). For instant, it has been shown that in Europe non-western emigrants are more prone to the development of vitamin D deficiency due to skin pigmentation or peculiarities of cultural clothing style and diet (45). Surprisingly, in Scandinavian countries the magnitude of vitamin D deficiency was less than in continental Europe, explanation is that in Nordic countries people tend to eat more oily fish and use supplementation such as cod liver oil. While in south population tend to use more sunscreens and developed the habits of avoiding direct sun exposure due to fear of skin cancer (48). As regarding Middle East and Asia it has been determined that for this population vitamin D status correlates with the clothing style. The worst vitamin D status was detected among veiled women and it improves when clothing changes towards western type (45). Also, it has been find out that population from Southeast Asia has more improved vitamin D status than in other parts of Asia, and it is correlated with consumption of vitamin D rich sea food. In South Asia the situation aggravated with low calcium intake and consequently vitamin D deficiency presented together with rickets, osteoporosis and fluorosis (49).

Moreover, recently global studies on vitamin D status have been conducted. The main idea behind global studies is to collect comparable data on vitamin D status among population in different countries by measuring it with one assay at one central laboratory. Study population for these studies were postmenopausal women with risk of osteoporosis from different continents. And the result in general confirms the known pattern. The best vitamin D status was determined among Canadians, next is population in US and furthers North Europe then Continental Europe and the worst condition in Middle East (45, 48).

1.1.10 Vitamin D status in South Asia

As it was discussed above, the vitamin D deficiency is prevalent around the globe, and South Asian region is no exception. Various reports (45, 47-50) have shown that the average circulated vitamin D level in adults in South Asian region ranges from 25 nmol/l to 50 nmol/l the same figures relevant to neonates and infants.

To the best of our knowledge, the little is known about vitamin D status among population in Nepal, during literature review, one article was identified on micronutrient deficiency of pregnant women during first trimester in rural Nepal (51). It has been demonstrated that the prevalence of vitamin D deficiency among pregnant women varied from 4.3% during summer hot season to 7.1%; 16.4%; and 24.4%; during fall, spring and winter seasons respectively. Another study conducted among alcohol-use-disorders inpatients recorded 64% of vitamin D deficiency defined as a vitamin D level less than 50 nmol/l (52).

Taking into consideration that there is a limited data on the vitamin D status in Nepal, farther discussion will be focused on the studies that have been conducted in South Asia but within the same geographic latitude. Although, most of the studies have explored vitamin D status among pregnant women and adolescent with convenient sampling methods, and consequently it affect representativeness of studies' results but still helps to identify general pattern of vitamin D status in the South Asian region. As related to vitamin D status of paediatric group, data concentrated predominately on the neonates and infants under six months.

Study from northern Indian (53) has shown high prevalence of vitamin D deficiency up to 84% among pregnant women from urban and rural population. The authors identified cut off point of 25(OH) D as a level below of that the PTH started to rise and for these study subjects it was 22.5ng/ml. Additionally, result has shown no differences between vitamin D status of urban and rural population, although it was assumed that rural population have better opportunities for sun exposure and consequently should have better vitamin D status.

Marwaha at al. studied vitamin D status among schoolchildren at age 10-18 years of old from two different socioeconomic backgrounds (54). The magnitude of vitamin D deficiency, qualified as a level below 20ng/ml, was surprisingly high 92.6% for LSES and 84.9% for USES. It is significant that subjects from the both groups had the same amount of sun exposure but different amount of calcium intake. Authors had concluded that this finding suggested possible effect of nutritional status on the vitamin D level (54). Nevertheless, it has not been proven by Puri at al. (55). Authors explored vitamin D status among schoolgirls and

possible association with socioeconomic status, it has been shown high prevalence of vitamin D deficiency in both strata, but even higher for USES 91.9% compare to LSES 89.6% and it was correlated with sun exposure but not with calcium intake.

In 2009 Sahu at al. published result from the cross-sectional study conducted during 18 months in India at latitude 26° N (56). It has shown high prevalence of vitamin D deficiency among pregnant women and adolescent girls 74% and 88.6% respectively, determined as 25(OH)D level below 50nmol/l. Although this study has limitation which was discussed by authors, in fact it was first study that identified prevalence among rural population.

Remarkably, is that authors identified seasonal variation of vitamin D status among population living at low latitude. Similarly, Marwaha at al. (57) identified tremendous high prevalence of vitamin D deficiency among pregnant women and their exclusively breast-fed infants in Delhi 96.3% and 98.8% defined as a level less than 50 nmol/l, and significant seasonal variation on vitamin D status through the year. Also, it has been shown positive correlation between vitamin D status of lactating mother and their newborns. Low average circulated level of vitamin D among term exclusive breastfeed infants and their mothers has been confirmed by Agarwal at al. in the study from Delhi (58). It has been identified maternal mean level of 25(OH)D 8.89 ± 5.97 ng/ml and 11.55 ± 11.7 ng/ml for infants at 10 weeks and 8.89 ± 5.97 ng/ml for infants at 6 months. Those results concur with the Pakistani study published in 1998 by M.Atiq at al. (59), authors reported mean level of 25(OH)D for under 6 months breastfeed infants equal to 24.74 ± 18.17 nmol/l and 49.97 ± 30.38 nmol/l for older ones. Table 5, summarize the variation of recorded vitamin D status in different population in the region.

Table5, Variation of serum 25(OH) levels in the South Asian region

Location	Latitude	Type of study	Population	Mean level 25(OH)D	Prevalence among study population	Reference
Pakistan, Karachi		Cross-sectional	Breastfeed infants(6weeks- 11months) and mothers	Age<6 months 24.74±18.17nmol/l Age>6 months 49.97±30.38nmol/l		Atiq at al. (59) 1998
India, Delhi		Cross-sectional	Healthy urban subjects, male and female	Pregnant (summer) 21.9±10.73nmol/l Newborn (summer) 16.72±4.99nmol/l Health personal(summer- winter) 17.98±7.98nmol/l 7.98±3.49nmol/l		Goswami (60) 2000
India		Hospital-based, case-control	Children aged 2-60 months, cases-ARTI, control-healthy		95% for cases 61% for control 25(OH)D less than 50nmol/l	Wayse at al. (61) 2003
Nepal	–	Data from population based trial	Pregnant women, first trimester	–	16,4%-4,3%-7.1% - 24,7% (from spring to winter) 25(OH)D<25nmol/l	Jiang at al.(51) 2005
India Delhi	28 ⁰ N	Cross-sectional	Schoolchildren 10-18 years, LSES/USES	LSES 10.4±0.4ng/ml USES 13.7±0.4ng/ml	35.7% 25(OH)D less than 9ng/ml	Marwaha (54) 2005
India	26,8 ⁰ N	Cross-sectional	Pregnant women and newborns	14.09±9.5ng/ml-for women 8.4±5.7ng/ml-for cord blood	–	Sachan at al.(53) 2005
India, Delhi	28,37 ⁰ N	Cross-sectional	Healthy school girls, 6-18 years LSES/USES	LSES 34.61±17.43nmol/l USES 29.38±12.69nmol/l	–	Puri and Marwaha at al.(55) 2007

India	26 ⁰ N	Cross-sectional	Pregnant women, adolescent girls (rural)	–	88.6% for girls 74% for women 25(OH)D<50nmol/l	Sahu et al. (56) 2009
India, Delhi		Cross-sectional	Middle-income male and female, 15-60 years	17.5±10.2 nmol/l	87% Less than 25 nmol/l	Goswami (62) 2009
India, Delhi		Prospective-cohort	Term exclusive breastfeed	Infants at 10weeks 11.55±7.17ng/ml At 6 months 16.96±133.33ng/ml Mothers 8.89±5.97ng/ml		Agarwal at al. (58) 2010
Bangladesh , north-eastern part		Case-control	Children aged 1-23 months, cases-ARTI, control-healthy	Cases 29±17.2nmol/l Control 39.1±9.4nmol/l		Roth at al. (23) 2010
India		Cross-sectional	Pregnant women and breastfeed infant	–	96.3% of women, 98.8% of infants 25(OH)D less than 50nmol/l	Marwaha (57) 2011
Nepal	28 ⁰ N	Cross-sectional	Alcohol-use disorder patient	43.9%±20 nmol/l	64% 25(OH)D<50nmol/l	Neupane at al. (52) 2013

1.2 Rationale for the study

This study is emerged on the following basis of

- Scientific gap

Despite, there are several strong reasons to anticipate the high prevalence of vitamin D deficiency among children in rural Nepal, there is a little scientific research in this area.

- Child Health perspective

The current appreciation of vitamin D's importance for child health and understanding that deficiency may cause not only rickets but also contribute to the magnitude of childhood illness and have serious implication for future life.

Thereby all these data lifted up the importance of identifying vitamin D status among children in Nepal.

1.3 Hypothesis

We hypothesized that despite of living in low latitude the magnitude of vitamin D deficiency and insufficiency among children at rural Nepal is high.

Hypothesis for the purposed research was based on several findings from the literature review. In fact, there are available data that indicates high prevalence of vitamin D deficiency among population in South Asian region.

1.4 Objectives

General objective: To assess the vitamin D status and contributing factors among children between 1 to 5 years old in Nepal.

Specific objectives

1. To quantify the level of 25-hydroxivitamin D₃ and D₂ among children from 1 to 5 years of old in a target setting.
2. To measure the prevalence of vitamin D deficiency and insufficiency among study population.
3. To calculate Calcium intake from milk products among study population.
4. To explore possible associations between vitamin D status and socio-demographic variables.

1.5 Introduction to study area

1.5.1 Geographical features

Nepal lies between latitude 26° - 31° N and longitudes 80° - 89° E in southern part of Asia and boards with China in north and Indian in south. The altitude range is from 70 m above sea level to 8,850 m (Mountain Everest).



Figure 2: map of Nepal (63)

Nepal is landlocked country with total area of 147, 187 sq km and consists of three physiographic belts: Terai, Central Hill region, Mountain in the north. This separation correlated with the altitude range, from 70 to 1000 metres for Terai area, 800-4000 metres for Hill belts, and Great Himalayan Range for the Mountain belt (63).

1.5.2 Climate

Nepal's climate varies from the tropical to arctic due to enormous ranges in altitude. Terain belt characterises with tropical summer and mild winter, in the Mountains climate changes towards cool and above altitude 3 500 metres it is actually arctic climate with severe winter. As concerns seasons, in general about of 80% of annual rainfall is during monsoon period from June to September when the weather is hot and humid. The average temperature in Kathmandu Valley during that period ranges from the min 19⁰ C to the max 29⁰ C; and from October to June is dry and cool season with temperature ranges from +2⁰ C during winter months to 30⁰ C for spring. Nepal has over 300 sunny days a year, with the average 8 sunlight hours per day from March to October (63).

1.5.3 Economy and society

As regards, the administration, Nepal is divided and subdivided into regions, zones, districts and Village Development Comities (VDC) respectively. Nepal has total population of 30, 430 267, the majority lives in rural areas 81%, in general 25.2 % of population lives below the poverty line. For the 75% of population the main employment is agricultural field. At the same time, GDP per capita is 1300 USD \$ (for comparison in Norway it is 55,300 USD \$). The vast majority of population are Hindu 80.6%, the rest is Buddhist, Muslim, Kirant and others are 10.7%, 4.2%, 3.2%, 0.9% respectively. The literacy rate is 60.3% measured as percentage of total population over 15 years that can read and write (64).

1.5.4 Health profile

The infrastructure of health system in Nepal is based on the administrative pattern of country.

Primary level: Sub Health Post, Health Post, Primary Health Care Centre; Secondary level: District Hospital, Zonal and Regional Hospitals; Tertiary level: Central and Teaching Hospitals (63). Nevertheless the coverage of health care system is low, and the majority of people in rural areas remain deprived of advanced health care facilities. This situation is aggravated with underdeveloped traffic and public transport system (64).

Table below describes some public health indicators, as comparison data from Norway are available.

Table 6: Public health indicators*

Indicators	Nepal	Norway
Life expectancy M/F	65,26/ 67,82	79/ 83
Fertility rate	2.36	1.77
Maternal mortality	170/100 000	7/100 000
Under five MR	50/ 1000	3/ 1000
Underweight children (under the age of 5 years)	29.1 %	
Prevalence of TB	238/ 100 000	8/ 100 000

*From CIA factbook. Mortality Ratio from WHO(65) .

2. Chapter II, Methodology

2.1 Overview

A community based cross-sectional study was conducted in Ugrachandi Nala Village Development Committee of Nepal. The data collection took place between the 29th of September and the 10th of November.

For the vitamin D assessment, blood samples have been collected using dried blood spot technique. The bio samples have been transported from Nepal to Norway for the farther analysis at the “Vitas” laboratory. Information regarding calcium intake and background variables has been collected through structured questionnaire.

2.2 Study design

Study design was chosen based on the objectives. The main objective of study was to assess the vitamin D status and contributing factors among children between 1 to 5 years old in Nepal. In other words, we wanted to know the prevalence of vitamin D insufficiency and deficiency among target population. For that aim it was reasonable to conduct a **cross-sectional** study with further **quantitative analysis** of the collected data.

2.3 Sampling

2.3.1 Study area and population

Ugrachandi Nala VDC belongs to Bagamati zone and is situated within Kathmandu Valley. It consists of nine wards (villages), with total population of 6327.

The choice of study site was based on several reasons. First of all due to geographic coordinates, the VDC is located at the latitude $27^{\circ}39'N$ and altitude 1400m. It has been shown by Holick et al. that the synthesis of vitamin D decreases significantly during winter months even at latitude $27^{\circ}N$ if altitude is below 3400m (43). Secondly, VDC is diverse in many ways; it consists of very remote, inaccessible wards as well as wards that are close to the nearest city Bonepa. Also, the population of the VDC belongs to different ethnic groups and socio-economic layers. All these factors make the Ugrachandi Nala VDC an appropriate area for the current study.



Figure 3: Ugrachandi Nala –study area

2.3.2 Sample size calculation

The sample size has been calculated based on the prevalence of vitamin D deficiency recorded in the rural Nepal at the latitude 26,96°N among pregnant women during first trimesters, which has been measured during winter months (51). The stated prevalence is lowest among those that have been identified during literature review in the region.

For sample size calculation we used the following formula

$$N = Z^2 P (1-P) / d^2,$$

Where: **Z** for 95% of CI is 1, 96; **d** is precision = 0, 05; **P** is expected prevalence 24, 4% (0, 24);

Based on the calculation above we have to include 280 children between 1- 5 years in order to show that vitamin D deficiency is prevalent in that area (24.4 %).

2.3.3 Inclusion criteria

All children between 1 to 5 years old in the chosen villages were eligible.

2.3.4. Exclusion criteria

_Child was excluded from the study if meets at least one of the following criteria:

- Known metabolic bone disease or chronic disease known to be associated with bone abnormalities (inflammatory bowel disease, celiac disease).
- Current medication is likely to interfere with vitamin D metabolism (Phenytoin, Phenobarbital, Carbamazepin, Isoniazid, Theophylline, Rifampin).
- Parents Inability to provide informed consent or comply with study protocol.

2.3.5 Sampling study subjects

So far as there is not available demographic registry in Nepal. We decided to make list of children from 1 to 5 years of old based on the available records. As a base for sampling we used records from Nepal's national vitamin A supplementation program for children aged 6 to 59 months with coverage of 90.4% as for 2011 (64) available at local health post. Out of available records we created numbered list of all 411 children aged 12-60 months living at VDC. By using online randomizer, a set of 320 unique sorted numbers was generated with range from 1 to 411 (representing the children assigned numbers). Subjects were selected randomly according to the produced list of numbers. Community Health Volunteers women were requested to identify selected children and invite guardians for participation in the study. We selected 320 children in order to achieve sample size of 280 subjects.

2.4 Research group at field work

- Research assistant, with bachelor degree in nursing and experience in research field work;
- Nurses, with 4 years' experience working in child health hospital; one permanent and one substitute nurse;
- We were cooperating with community health volunteer's women from each ward during recruitment stage.
- Principal investigator with 3 years of experience working in child health clinic as a staff paediatrician;

2.5 Training for research assistant and nurses

The vitamin D physiology, determinants for the development of deficiency and importance it for child health has been presented on the lecture for the research assistant and nurses. Also, it was explained objectives of the study and information stated in the consent form and how to treat participants.

Additionally, research assistant was taught, how to conduct interview, all points in the questionnaire have been discussed separately, with possible answers, and how to record them. The procedure of data entry from questionnaire to EpiData has been explained in later stage. The importance of preserving confidentiality has been outlined.

Nurses has been taught regarding procedure of taking anthropometric measurements, according to WHO Training course on Child Growth Assessment (66), she was explained how to do it correctly and record accurately. Also, collection of blood spots for the vitamin D analysis was described, through watching educational film and leaflets, provided by Vitas laboratory in Norway. All stages of dry blood spots collection technique have been emphasized.

2.6 Pilot study

Pilot study has been conducted among first 10 households at ward number 3. The aims of pilot study were the following: To pre-test the questionnaire, make sure that each question is understandable and the possible answers are mutual exclusive. To quantify time needed for one interview and to calculate the maximum number of participants per day. To identify the general flow of data collection,

Based on the result the following changes have been made:

- The question regarding diet (mothers were required to describe what would typically child have for breakfast lunch or dinner), we found that all families provide the same traditional meal for children from 1-5 years of old, so the question was considered not informative.
- The question related to the assessment of child's gross motor and teething milestones was omitted, because majority of parents were not able to recall information accurately.
- The question regarding child illness (the mothers were requested to specify the severity of ARTI during the last 4 weeks, if any) we found that it is not clear enough, so we modified it with possible one or more options (child was playing/ lying in the bed/ refused the meal or breastfeeding/ breathing difficulties/ did you call health personnel)
- At first, according to study protocol it was planned to take blood samples by pricking the children's heel if they are smaller than 2 years of old, and for older ones from the finger. But we observed, most of the children do not wear appropriate shoes or socks what might cause the contamination of wound after puncturing. So, it was decided to take blood samples by pricking the tips of finger for all age groups.

- Initially was planned to conduct data collection from household to household, however during pre-testament it became clear that it is not convenient. The reasons were that it was not possible to find firm floor and straight wall for adjustment of anthropometric equipment, or enough space to work at most of the households. Also, it was difficult to find child and mother at the same time at home. So, we decided to conduct data collection in one or two places for each ward, with the convenient location for the local community and suitable for the research group.

2.7 Data collection

2.7.1 Recruitment

The community healths volunteers (CHV) were informed about the objectives of the study, and were asked voluntarily cooperate with the research group.

Ten CHV' women from nine wards have been contacted to identify 320 selected children. 316 children have been identified. Out of 320 children, 11 have been moved permanently, 9 children were away, guardians of 16 children refused; as a result the 280 children have been recruited for the present study. Additionally, 13 children out of randomly created sample were included to the study by the request of the guardians.

The place and date for the data collection has been discussed priory with the community leaders of each wards based on the harvesting schedule in the village and daily routine of the community. The invitation to the guardians of chosen children has been send by the CHV via phone call or door to door visit two days before of assigned date for the each ward.

Consequently, the main data collection took place during 11 days at different public or private places, such as schools outside of working hours or community health worker's household. For those who were not being able to participate in regular day, we arranged household visit, by their request.

2.7.2 Data collection flow

In general, data collection was organised in three stages. The objective of the study and data collection was explained for the guardian. After informed consent form has been signed, the research assistant fulfilled form with personal information of the child. Consequently each child has been encoded with the random identification (ID) number. Only code number was used during study, and the key between code and candidates' ID was kept separately to ensure confidentiality. The form with personal information, questionnaire and card for blood sample has been coded with the same number for each child. We have used prepared stickers with the same ID numbers on the group of three labels for each child to avoid confusion. The unique ID numbers has been generated randomly by software and consisted of two capital letters and three figures.

At first, mother was interviewed according to structured questionnaire with research assistant. At second, anthropometrics measurement of the child has been taken by nurse with the help of mother. At third, collection of blood samples from the child.

At the end of each data collection day, all forms that have been used were tallied with sample cards by similarities of identification number.



Figure 4: Interview , during data collection at ward № 8, public school

2.7.3 Interview

For background variables and food frequency assessment we have used structured questions. The questionnaire were prepared in English and translated into Nepali language. The general structure and the question's number remained as in the original version. The questionnaire consisted of the following sections: household information; infant feeding history; child's diet, consumption of milk's products; immunization history; child's development; sun exposure; The interviewer was reading the questions and recoding answers. At the end of interview, questionnaire was checked for the any missing data or inconsistency. For the question required understanding of measurement scales (millilitre of milk) we demonstrated the common kitchen bowl, which normally used in the population for the child's feeding.

Milk products intake was calculated by multiplying the amount per serving and the frequencies of consumptions for each type of milk product. Calcium intake was computed by

multiplying the calcium content of the specific type of milk by the daily consumed amount. Total calcium intake from milk products was calculated by summing the calcium intake from different milk products for each child. Calcium value in milk products were obtained from USDA National Nutrient Database for Standard Reference (67) . All calculation related to Calcium consumption has been done during data analysis stage, during data collection just consumed amount of products has been recorded.

2.7.4 Anthropometric measurement

Anthropometric measurement were performed according to WHO Training course on Child Grow Assessment (66). Weight has been measuring with digital weight scale made by Microlife Company in Swiss, certified for ISO 9001 accurate to 100g. Height has been measuring by wall stadiometer made by Bio-Plus 200cm, Model 265M/1013522.

Place for the anthropometric measurement has been chosen according to the following criteria: sturdy, flat surface for the weight scale and strong, straight wall to hang stature meter. The measurement were taken according to WHO Training course on Child Grow Assessment (66). The clothing on the child has been minimized; shoes have been taken off before the measurement. Each child has been weighed twice; we recorded results of both measurements if they were different, afterwards the mathematically average were calculated. For the children under 24 months or with difficulties to stand alone on the scale we weighed mother and child simultaneously and calculated child's weight mathematically based on the two different measurements, by subtracting weight of mother from the weight of mother weighted with child. Height was measured by stature metre hanged on the wall to the nearest 1 mm. During measurement child was requested to look straight ahead at the mother (standing in front of him/her). Mother was asked to assist by the fixing child's shins and knees. The nurse made sure that the shoulders are level at the same time fixed the chin and performed measurement. For a few cases when child was scared and extremely uncomfortable the height has not been measured. The result of weight and height measurements has been recorded in the questionnaire of each participant.

2.7.5 Assessment of nutritional status (has been done during data analysis)

The nutrition status of the children was determined by comparing measurements of weight for height, weight for age and height for age to the WHO reference standards for wasting, underweight, and stunting indicators, respectively (68).

1. weight-for-height (WFH) – wasting indicator (acute or recent malnutrition)
2. weight-for-age (WFA) – underweight indicator (acute or chronic malnutrition)
3. height-for-age (HFA) – stunting indicator (chronic or long-standing malnutrition)

Global acute malnutrition (wasting) is defined as <-2 z scores weight-for-height and/or oedema. Severe acute malnutrition is defined as <-3 z scores weight-for-height and/or oedema. Underweight is defined as <-2 z scores weight-for-age while severe underweight is defined as <-3 z scores weight-for-age. Chronic malnutrition (stunting) is defined as <-2 z scores height-for-age while severe chronic malnutrition is defined as <-3 z scores height-for-age.

2.7.6 Assessment of vitamin D level (collection of blood samples)

For the vitamin D assessment we have used whole dried blood spots technique with liquid chromatography- tandem mass spectrometry method. The LC-MS/ MS from DBS method was internally validate, it was chosen as a less invasive and convenient to use with paediatric groups in the field research. The vitamin D kits were provided by Vitas laboratory in Norway. Each kit consisted of paper card with marked circles for the blood absorption, foil zip bag with sachet of drying agent, swabs with alcohol, dry sterile swabs, single use lancet and plaster.

The nurse made sure that the child's hands were washed with soap and dried (soap, clean water and towel have been provided). Consequently, the card was opened and child's fingertip was wiped with alcohol swabs. Fingertip was punctured by single use safety lancet. After the prick, the first blood's drop was removed with sterile swabs and the next droops were applied directly on the sampling paper (card) with pre-marked three circles, one-two drop for one circle.



Figure 5: Nurse takes blood samples, ward №4

2.8 Data management

2.8.1 Storage and Transportation of blood samples

The cards were dried during 2 hours in the prepared trays and covered with medical gauze; afterwards sample cards were stored in low gas –permeable zip lock bag with desiccant packages. During first 7-10 days sample cards have been kept in a room temperature.

Consequently the samples were transported to the Bhactapur Cancer Hospital in order to be kept in the refrigerator under the $+4^{\circ}\text{C}$ with permanent electricity supply until the end of the data collection.

At the end of field work bio samples were divided into two parcels and shifted to the Vitas laboratory in Norway by DHL Express. The samples were sent in two separate parcels with

two day differences processing from the security reasons, and were received in three days each. The levels of 25(OH) D₃ and 25(OH) D₂ were quantified separately by using liquid – chromatography – tandem mass spectrometry (LC-MS/ MS) from dry blood spots.

2.8.2 Data handling and entry

At the end of each data collection day, all forms that had been used were tallied with sample cards by similarities of ID number. The bio materials were stored as it is described above. The questionnaire and personal information form were safely kept at the locked room.

Double data entry was done during two weeks after the data collection stage had been completed. The data was entered to EpiData by principal investigator and research assistant. Two types of file were created, one for personal identification information another for questionnaire. The random ID number was marked as key variable for both files. The missing data was recorded as 99 and not applicable one as 88. Consequently, two sets of files had been compared, whether data in two different fillies is the same, and all recorded discrepancies that occurred due to mistyping were corrected. When result from vitamin D test had been available, it was entered based on the key variable.

The questionnaires were sent by post, all personal information and consent forms were brought by hand carriage to Norway. Then, all set of data were kept at the locked cabinet at the Department of Community Medicine.

2.8.3 Coding variables and preparing data for analysis

The data were transformed from EpiData into PAWS, version 20. In general, we had 45 variables, the majority of our variables were categorical, when fixed number allocated to the specific answer. Additionally, we created some new categorical variables from the continuous numerical, such as ‘monthly household income’ It was done to identify three levels of possible salary: low 0-14999 NR, middle 15000-39999 NR, and high 40000 NR or higher;

Consequently the data was screened for the missing data by using frequencies for categorical variables and descriptive for continuous numerical; outliers and normal distribution was assessed through using descriptive statistic and creating box plots and histogram;

2.9 Data Analysis

Statistical analysis of the data was performed by using PASW software (version 20, SPSS IBM Corporation, USA). WHO Anthro software (version 3.2.2), was used to evaluate gender specific growth indicators in relation to WHO standards (68). Standing height for children younger than 24 months was equalized to the recumbent length by adding 0.7 cm. according to WHO Anthro software's guidelines. Data from descriptive statistic were presented as mean scores and standard deviation. To compare the mean scores of continuous variables between two different groups of categorical variables we used independent sample t-test (two-tailed). One way analysis of variance was performed to explore association between vitamin D concentrations with the categorical explanatory variables. For unequal variances the non-parametric test has been used (Kruskal- Wallis). Results have been considered statistically significant with p value below 0.05.

2.10 Timetable

Protocol writing and research planning – April- June, 2012

Time allocated for field work 3 months- September- November, 2012

- Meeting with CHV and community leaders -1 week
- Translation of questionnaire into Nepali language – 1 week
- Training for research assistant and nurses - 1 week
- Pilot study 1 week
- Changes in the questionnaire and discussion with research team – 1 week
- Data collection – 6 weeks
- Data entry – 2 weeks
- Miscellaneous activities – 1 week

Laboratory analysis of bio material –December-June, 2013

Thesis writing – February- July, 2013

Paper submission – November, 2013

Thesis submission- November, 2013

2.11 Ethical issues

The study was approved by the Nepal Health Research Council and Regional Committee for Medical and Health Research in Norway. Informed written or witness consent was obtained from the guardians of all participants. Witness consent was acquired when guardian had a decision making capacity but cannot read or write due to illiteracy. Witness was an adult from the same community who was not a member of study team.

On the early stage of study planning we have identified several vulnerabilities of our study subjects. One of factors that make the general population vulnerable is poverty and high illiteracy rate. Another aspect was that the study units are children, depended upon their guardians. Moreover there were imbalance of power between population and research group, due to our cooperation with community health volunteers' women for the recruitment procedures.

Proceeding from the factors mentioned above we assumed the following measurement to ensure that the study conducted according to the international ethical guidelines for research involving human subjects as it is stated in the Declaration of Helsinki.

- The inform consent form was read by research assistant, and additionally explained in a simple manner, the possibility to withdraw child from the study at any stage of research work was emphasised.
- During the data collection, interviewer took into the consideration that the interview take place in the public place with other people around. So, before asking sensitive question, the possibility to omit the question was emphasised.
- As regards, the blood collecting procedures. In the cases when child was extremely uncomfortable and expressed it with cry, we made sure that mother still want to take part in the study. The opportunities of withdrawal child at all or participation in the other day was explained. Additionally, we prepared small gift for each child to compensate uncomfortable feeling from the painful procedures. The gift package

consisted of school supplies and personal hygiene items, the cost of each pack was around 100 Nepalese Rupees, we thought that this small amount would not be considered as an inductive method (1 Nepalese Rupees as of April 1, 2013 = 0.107 NOK) but will give pleasant feeling to the child.

- We were cooperating with community health volunteers' women during recruitment procedures. As a matter of fact it made possible to keep random sampling. However, we undertook all cautions to avoid misunderstanding from the local population regarding involvement of CHV. The CHV were instructed about voluntarily bases of study, and what should include message with invitation towards participants. Before giving agreement to take part in the study participants were explained that there is not any association between current study and their relationship with CHV. In other words, it was emphasised that they are free to make decision whether to take part or not and it will not have influence on any farther services that they might need from CHV.

2.12 Methodological consideration

Study was designed as a community based cross-sectional. Cross-sectional study is ideal for describing of variables' magnitude in our case the prevalence of vitamin deficiency and insufficiency, as well as to explore the association between vitamin D status and background variables. However, the cross sectional design estimate outcomes and exposure in the same time, consequently the chosen methodology does not allow us to look into causality of vitamin D deficiency or to clarify which factors contribute more to the development of vitamin D deficiency as it do in a *case- control design*. Another limitation of chosen methodology is inability to gain knowledge about consequences of vitamin D deficiency through the making follow up as it is possible in *cohort study*.

Nevertheless, the cross-sectional design meets objectives of study. Also, based on the literature review, we can say that the vast majorities of relevant studies that make a framework for our purposed research had a cross – sectional design.

2.12.1 Strength and weakness that effect on internal validity

Strength and limitation discussed on the light of assessing quality criteria for prevalence study. Internal validity can be affected with introduction of bias from sampling and recruiting participants to collecting data and performing measurement. In our study we identified selection and information biases, those was not possible to avoid due to particularities of research setting.

Sample size and data collection period

Sample size was calculated based on the high precision d equal to 0.05, it reduces the width of CI to 10%, and we reached whole sample size as it was planned. Although in order to calculate sample size we had used lowest prevalence recorded during winter months from the South Asian region that was identified during literature review. But, due to framework of master program, it was not visible to conduct field work during winter months; our data collection was done from 29th of September to 10th of November.

Sampling method and selection bias

For the drawing sample from population the randomization was applied. In other words all children got the equal chances to be selected for the study. Although, due to the absence of demographic data in the region, we have had to create our own databases from the records of vitamin A supplementation program, available at VDC health post. So, children those for some reasons did not get vitamin A during last 6 months were not included in our databases, and consequently did not get chance to be selected for the current study. This kind of selection bias may result that our sample more representative for those who has better access to health care programs than for general population in total.

Response rate

In the current study we got respond rate 87.5%, which is considered adequate for the prevalence study. Nevertheless, we do not have information on non-respondents. It might be that people who did not give consent are different in their lifestyle, health seeking behaviour or in other ways from the participants.

Measurements with valid instruments

Before actual data collection, pilot study was conducted to pre-test the questionnaire, and the necessary changes were done. Nevertheless, there were other limitations of the study: Information was gained through self-reported questionnaire with help of interviewer and it may affect validity of data regarding child's health or nutrition. It is possible that parents might over report use of milk products or completeness of immunization history due to socially acceptable meanings of what is best for the child. Also, we observed that parents tend to under report information on respiratory infection. We assumed that it had happened due to culturally different determination of respiratory infection. It would be more advisable to conduct small qualitative study beforehand with aim of exploring the meaning of respiratory infection in the local community and based on that design questionnaire. Also, the question regarding sun exposure was not enough detailed, it did not clarify time of being outside in the

relation of noon. It is important due to the effect on the vitamin D production in the skin under the impact of UVB.

Additionally, it was not visible to allocate separate place for the interview. Which may have resulted response information bias since, participants might be shy to give answer on the question regarding income level or drinking problem in the family. Another source of information bias was language barrier, it was not possible to ensure that research assistant was asking question and recording answers in the same manner in the relation to all participants as we assumed.

Another aspect that affects the quality of data is approximation in the age of children. Nepalese people did not register children immediately after birth, situation with clarifying an age was aggravated because of differences in calendar year between lunisolar Hindu calendar officially used in Nepal and western Gregorian one. So, we register age of child based on the mother's claiming, but we accept deviation in 6-12 months. Yet another limitation, for weight and height measurements we used equipment that were not standardised for establishing of anthropometric databases.

Assessment of vitamin D status

For the assessment of vitamin D status we have used dried blood spot sampling technique with farther analysis of 25(OH)D by using LC-MS/MS methods. LC-MS/MS methods allows to measure 25(OH)D₂ and 25(OH)D₃ separately and is considered as a gold standard for the assessment of vitamin D level(35).As regarding use of dried blood spot techniques for the blood collection it has been shown good agreement with the serum level (39) and was internally validated in the laboratory. DBS sampling method allowed us to avoid venepuncture for children and consequently to follow community based study design and to achieve whole sample size. Blood samples were collected, stored and shipped according to recognized standards.

It might be considered as limitation the absence of data on the level of calcium, phosphorus and alkaline phosphatase from our study population. Although, these indicators deviate during severe vitamin D deficiency and are more relevant to the assessment of bone metabolism and (40). However, simple measurement of 25(OH)D reflects the objectives of our study to assess the vitamin D status of children.

2.12.2 Strength and weakness that effect on external validity

High degree of external validity means that the result of the study can be applied to population from which it drawn. External validity is determined by the representativeness and homogenous of the group presented in the sample. In general, this study is unique in terms of study subjects and setting, it was designed as a population based so it has better representativeness than those that were designed as a hospital or school based. Although, as we discussed above, children in our study represent those who has some access to health care program in rural area of Nepal at latitude 27,39⁰ N. However, thinking about representativeness, we should take into consideration that Nepal is country with tremendous diversity of altitude. In fact, altitude effects on the vitamin D production through the whole year. Consequently, we can conclude that result of present study could be generalized in certain extend with respects to geographical coordinates and study population.

2.13 Disseminations of Results

Present research work was conducted as a part of M.Phil. Program in International Community Health, consequently thesis was submitted to the Department of Community Medicine at the University of Oslo. The thesis will be available online on the DUO system at the University Library. Besides, an article was written based on the data from the field work, co-authored with supervisor, co supervisor and collaborative laboratory. Article was submitted to the *British Journal of Nutrition*.

Copy of thesis and article after publication will be send to NHRC, as we claimed in the application form. The results of vitamin D tests will be available for guardians of all children as it was stated in the protocol. It will be send electronically to research assistant, who will make it available at the local health post.

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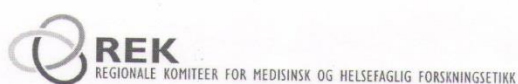
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4 Appendices

Approval from the Regional Committee for Medical and Health Research Ethics North Norway



Region: REK nord	Saksbehandler: Veronica Sørensen	Telefon: 77620758	Vår dato: 14.08.2012	Vår referanse: 2012/1008/REK nord
			Deres dato: < Sett inn dato til inngående skjema/e-post her >	Deres referanse:
Vår referanse må oppgis ved alle henvendelser				

Ahmed Madar

ISAM
pb 1130
0318 Blinder

The vitamin D status among children 1 to 5 years old in Okhaldhunga district of Nepal
Our reference 2012/1008

Chief Investigator's research project description

The prevalence of vitamin D deficiency and insufficiency is high worldwide and South Asia is not exception despite of the plenty of sunshine. The main objectives : To assess the vitamin D status among children 1 to 5 years old in Nepal. Design: cross-sectional community based study. Methods: laboratory assessment, the level of 25(OH)D2 and 25(OH)D3 will be measured in whole dry blood spot by using liquid chromatography tandem mass spectrometry methods; the food frequency questionnaire will be used for calculation of dietary calcium and vitamin D intake. Short structured interview will be performed to collect data regarding background variables. Expected outcomes: we aim to identify the prevalence of vitamin D deficiency and insufficiency in rural Nepal among children age of 1 to 5 years old. b. Research

This is to confirm that the Regional Committee for Medical and Health Research Ethics North-Norway (REK nord) has approved the project.

Letters from REK are approved transmitted electronic without signature.

Sincerely,

May Britt Rossvoll
secretariat leader

Veronica Sørensen
executive officer

Kopi til:

Besøksadresse:
TANN-bygget Universitetet i
Tromsø 9037 Tromsø

Telefon: 77646140
E-post: rek-nord@fagmed.uit.no
Web: <http://helseforskning.etikkom.no/>

All post og e-post som inngår i
saksbehandlingen, bes adressert til
REK nord og ikke til enkelte
personer

Kindly address all mail and e-mails
to the Regional Ethics Committee,
REK nord, not to individual staff

Approval from Nepal Health Research Council



Nepal Health Research Council

Estd. 1991

NHRC

Ref. No. 354

Executive Committee

Executive Chairman

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Ministry of Finance

National Planning Commission

Ministry of Health & Population

Chief, Research Committee, IOM

Chairman, Nepal Medical Council

23 September 2012

Ms. Diana Avagyan
Principal Investigator
University of Oslo, Norway

Ref: **Approval of Research Proposal** entitled **The vitamin D status among children from 1 to 5 years old in Urgachandi Nala Village Development Committee of Nepal**

Dear Ms. Avagyan,

It is my pleasure to inform you that the above-mentioned proposal submitted on 24 July 2012 (**Reg. no. 100/2012** please use this Reg. No. during further correspondence) has been approved by NHRC Ethical Review Board on 14 September 2012 (2069-05-29).

As per NHRC rules and regulations, the investigator has to strictly follow the protocol stipulated in the proposal. Any change in objective(s), problem statement, research question or hypothesis, methodology, implementation procedure, data management and budget that may be necessary in course of the implementation of the research proposal can only be made so and implemented after prior approval from this council. Thus, it is compulsory to submit the detail of such changes intended or desired with justification prior to actual change in the protocol.

If the researcher requires transfer of the bio samples to other countries, the investigator should apply to the NHRC for the permission.

Further, the researchers are directed to strictly abide by the National Ethical Guidelines published by NHRC during the implementation of their research proposal and submit progress report and full or summary report upon completion.

As per your research proposal, your research amount is US\$. 10,655.00 and NHRC processing fee is US\$ 299.00.

If you have any questions, please contact the research section of NHRC

Thanking you.

Sincerely Yours,

Dr. Shanker Pratap Singh
Member Secretary

Tel. +977-1-4254220, 4227460, Fax: +977-1-4262469, RamShah Path, P.O. Box 7626, Kathmandu, Nepal.

Website: <http://www.nhrc.org.np>, Email : nhrc@nhrc.org.np

Declaration of Consent

We cordially invite you to participate in a research project entitled “The vitamin D status among children 1 to 5 years old in Urgachandi Nala Village Development Committee of Nepal”.

The main objective of the project is to investigate the prevalence of vitamin D deficiency and insufficiency among children 1 to 5 years old in rural Nepal.

Vitamin D among other nutrients is important for the child growth and development. This study is necessary to identify the status of your child in this term, and useful for future intervention also.

In case you agree to give permission for participation of your child, child will be test for vitamin D level free of charge. Besides, we will send the results of the test to the local health post which can be collect when available, if it is found that your child has any insufficiency, the recommendation for the supplementary therapy will be given. In addition, the information gathered in the study will provide the foundation to improvements in the preventive strategies of vitamin D deficiency among children, resulting in reduced morbidity.

The examinations and investigations that will be carried out in the study (blood drawing to assess vitamin D level; questionnaires to assess socio-economic, and health data; anthropometric measurement) are not life-threatening. We shall ask you some questions that may take about 15-20 minutes. We will take measurement from child (weight and high). We would like to collect about 1ml (a little in Nepali) of blood using a needle prick on the child's finger or heel, a common procedure and does not pose a serious danger. Blood samples will be collected by qualified and trained health personnel, and precautions will be made to prevent infections and / or injuries. The samples and data that are registered about you will only be used in accordance with the purpose of the study as described above.

All information obtained from you will be strictly confidential. Participation is voluntary and you are free to refuse to participate in any procedure or to refuse to answer any question at any time without prejudice or consequences for further treatment. You are also free to withdraw your consent from the study at any moment. In this case, the blood sample of your child will be destroyed and the information you have provided will be deleted from the register. In addition, the research investigators will answer any of your questions about the

research procedures, your rights as a subject and research-related injuries. A copy of this consent form can be given to you if you so request.

Note: If you have any questions or complaints about the informed consent process or policy, please contact the investigators.

Based on the information provided, if you want to join the study, please sign below:

Place and date

Signature of the Participant

Signature of the Witness (In case the participant cannot sign)

Questionnaire for research project 'The vitamin D status among children from 1 to 5 years old in Ugrachandi Nala Village Development Committee of Nepal'

DATE <dd/mm/yyyy>

RANDOM ID

Respondent relation to the child _____

Household information

- Q001 Ward _____
- Q002 Religion _____
- Q003 Ethnicity: _____
- Q004 Number of persons in the household: _____
- Q005 Number of children from 1 to 5 years old in the household _____
- Q006 Sex (of participants/child) **M / F**
- Q007 Date of the birth of child/age in months _____
- Q008 Father's occupation _____
- Q009 Mother's occupation _____
- Q010 Type of the housing _____
- Q011 For how many months per year does your farming feed the family? _____
- Q012 Monthly household income (salary, sale of crops, etc. NRS)

Infant feeding

- Q013 Has (child's name) ever been breastfed? **Yes / No**
- Q014 If yes, Is the child still being breastfed now? **Yes / No**
- Q015 If yes, how often do you breastfeed your child now?
times /day _____
- Q016 If not breastfed anymore, how old was the child when you stopped breast-feeding completely? _____
- Q017 How old was your child when you gave him/her first solid food (porridge (LITO / JAULO)) _____

Vitamin and mineral supplementation

Q018 Did you (mother) take any vitamin or mineral supplementation during pregnancy?

Yes / No / Do not remember

(for those who breastfeed child now)

Q019 If Yes, please specify when, what type how often and how long? During _____ trimester, during _____ months

<u>If yes, please specify</u> Type of supplementation:	times/week 1 2 3 4 5 6 7 times/day 1 2 3	Amount per time 1 tab 2 tablet
-----------------------------------------------------------	-------------------------------------------------------	---------------------------------------

Q020 Do you (mother) take any vitamin or mineral supplementation during breastfeeding? **Yes / No / Do not remember**

(for those who breastfeed child now)

Q021 If Yes, please specify when, what type how often and how long? During last _____ weeks/ months

<u>If yes, please specify</u> Type of supplementation:	times/week 1 2 3 4 5 6 7 times/day 1 2 3	Amount per time 1 tab 2 tablet
-----------------------------------------------------------	-------------------------------------------------------	---------------------------------------

Q022 Does your child take vitamin and/or mineral supplements now? **Yes /No**

Q023 If yes, please specify type of supplementation:

Have been taking during _____

If yes, please specify Type of supplementation:	times/week							Amount per time	
	1	2	3	4	5	6	7	1 tab	2 tablet
	times/day							3ml	5ml
	1	2	3						

Q024 Immunization history (Information from health post's records)_____

Child's development

Q025 Please list at what AGE (month) that your child was with respect to the following

- 25.1 Sat up on their own
- 25.2 Started crawling
- 25.3 Stood with support
- 25.4 Stood on their own
- 25.5 Started walking
- 25.6 Walked up/down stairs
- 25.7 Started teething

Past and current health concerns, Please, indicate and give details

Q026 Childhood illnesses **Yes/ No**

If yes, please specify_____

Q027 Any chronic illness or disability **Yes/ No**

If yes, please specify_____

Q028 Accident **Yes/ No**

if yes, please specify_____

Q029 Major fall or injuries **Yes/ No**

if yes, please specify_____

Q030 Operation **Yes/ No** If yes, please specify_____

Q031 Hospitalization **Yes/ No**

If yes, please specify _____

Q032 Medication **Yes/ No**

If yes, please specify _____

Q033 Has child ever had convulsions without fever **Yes/ No**

If yes, please specify, how many time and when

Q034 Has child had diarrhoea in the last four weeks

Yes/ No

If yes, please specify how often and when was the last episode

Q035 Has child had symptoms of acute respiratory tract infections during last four weeks

Yes/ No

If yes, please specify how many times and how severe it was_____

- ☐ He/she was playing
- ☐ He/ she was lying in the bed
- ☐ He/she refused the meal/ breastfeeding
- ☐ Did she/he have breathing difficulties? Yes / No
- ☐ Did you call for health person? Yes/ No

General information on Diet

Q036 Please list what your child would typically have for: (question was deleted after pre-testing)

Breakfast	
Lunch	
Dinner Snacks	

Q037 How many times does your child have egg during his/her meal

_____ week (if never, leave it empty)

Q038 Is any specific diet regime followed? vegetarian / vegan / other

Q039 Other information on his/her

nutrition:_____

Q040 **Type of milk**

Type of milk	Per day	Per week	Amount of milk usually consume per time?
Standard milk formula	5+ 4 3 2 1 <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	1-3 4-6 <input type="checkbox"/> <input type="checkbox"/>	60 120 180 240 A B C D <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>
Whole milk purchased in the market (packet milk)	 <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	1-3 4-6 <input type="checkbox"/> <input type="checkbox"/>	 <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>
Cow milk	 <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	1-3 4-6 <input type="checkbox"/> <input type="checkbox"/>	 <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>
Buffalo milk	 <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	1-3 4-6 <input type="checkbox"/> <input type="checkbox"/>	 <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>
Goat's milk	 <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	1-3 4-6 <input type="checkbox"/> <input type="checkbox"/>	 <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>
Sour milk/yogurt	 <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	1-3 4-6 <input type="checkbox"/> <input type="checkbox"/>	 <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>

Sun exposure

Q041 How much time the child spends in the sun every day

<input type="checkbox"/>	< 15 minutes per day
<input type="checkbox"/>	15-30 minutes per day
<input type="checkbox"/>	more than 30 min- to 60 minutes per day
<input type="checkbox"/>	more than 1- 2 hours per day
<input type="checkbox"/>	> 2 hours per day

Q042 Describe, what does child wears normally outdoor?

- ☐ Trousers
- ☐ Short pan
- ☐ T-shirt
- ☐ Long sleeves shirt
- ☐ Cap/ Hat

Q043 Information on smoking

Does anyone smoke in the home? **Yes / No/ No answer**

if yes please specify_____

Q044 Information on alcohol consumption

Does anybody in the family have drinking problems?

Yes / No/ No answer

If yes, Can you specify? _____

Q045 Additional information_____

Q046 High (cm)_____

Q047 Weight (kg)_____

5. Paper

Vitamin D status among pre-school children in rural Nepal determined by using dried blood spot sampling.

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Running title: Vitamin D status-children-Nepal

Abstract

Insufficiency of vitamin D, which plays a major role in the calcium and bone metabolism, is reported from populations worldwide. Its extra skeletal benefits are being identified, vitamin D is important in function of immune and cardiovascular systems. However, little is known about the vitamin D status among populations from developing country settings, particularly in children. We aimed to assess the vitamin D status and contributing factors among children between 1 to 5 years of age in rural Nepal at latitude 27.39° N. A total of 280 children aged between 1 and 5 years were randomly selected from the records of vitamin A supplementation program and included in the study. Blood samples were collected using the dried blood spot technique. The level of serum 25-hydroxyvitamin D₃ (25(OH) D₃) and D₂ (25(OH) D₂) were measured in whole dried blood spots (DBS) by using liquid chromatography- tandem mass spectrometry analysis (LC-MS/MS). For the assessment of calcium intake and background variables we used structured questionnaire. Hypovitaminosis D, defined as a 25(OH)D₃ level less than 50nmol/l was identified in 91.1% of children. There was no association between level of 25(OH) D₃ and gender, socio-economic indicators, sun exposure or nutritional status. Vitamin D deficiency is common among children from 1-5 years of old living in rural Nepal.

Keywords: Vitamin D deficiency, Dried blood spot, Child health, South Asia

Abbreviation: liquid chromatography- tandem mass spectrometry (LC-MS/MS); dried blood spots (DBS); 25-hydroxyvitamin D₃ (25(OH)D₃), 25-hydroxyvitamin D₂ (25(OH)D₂)

Vitamin D is a prohormone that plays an essential role in the mineralisation of bones ⁽¹⁾. There are two ways for humans to meet requirement for vitamin D, the major amount is produced in the skin after exposure to sunlight and the rest is to be fulfilled through dietary sources. There are few foods that are naturally rich with vitamin D, although there are some fortified products available for consumption ⁽²⁾. Vitamin D deficiency leads to comprehensive failure in the mineralization of the bones and development of osteomalacia; for children when skeletal system is immature it manifests in the form of rickets ⁽³⁾. Moreover, the role of vitamin D as a prohormone for the human health is reappraised ⁽⁴⁻⁷⁾. It has been suggested that maintaining vitamin D level on the normal ranges is not only essential for normal development and function of skeletal system but also may play important role in the prevention of autoimmune, neoplastic and cardiovascular diseases ⁽⁸⁾. There is growing evidence that vitamin D deficiency may contribute to the incidence and the severity of acute lower respiratory tract infections as well as progression of tuberculosis to its active form ⁽⁹⁻¹²⁾. The nuclear vitamin D receptor is presents in the majority of human's tissues ⁽⁸⁾, and the most type of cells are able to transform vitamin D into the its active form 1,25-dihydroxyvitamin D ⁽¹³⁾. Hence, vitamin D regulates expression of more than 200 genes with transcription of various proteins. These proteins adjust cellular differentiation, proliferation and apoptosis with consequent effects on the function of many organs and systems ^(10, 14). As a matter of fact, for paediatric groups of developing countries the potential modular effect of vitamin D on immune system and on risk of acquiring respiratory infections is more crucial and influential on public health indicators.

Vitamin D insufficiency and deficiency are prevalent around the globe ⁽¹⁵⁻¹⁷⁾, it has been calculated that around 14% of the world population are vitamin D insufficient or deficient ⁽⁶⁾, even though the magnitude varies between age groups, gender and geographic region ⁽¹⁵⁾. During recent years, numbers of studies have shown high prevalence of vitamin D deficiency among South Asian population ^(15, 18). Various reports ^(16, 17, 19) from India show that the average circulating vitamin D levels in adults ranges from 25nmol/l to 50nmol/l the same figures relevant to neonates and infants ⁽²⁰⁻²²⁾. The little is known about vitamin D status among population in Nepal. There is available data regarding vitamin D status of pregnant women during first trimester, author recorded seasonal variation of vitamin D deficiency from 4.3% to 24.4 % with cut of level 25nmol/l ⁽²³⁾. Another study conducted among alcohol-use-

disorders inpatients recorded 64% of vitamin D deficiency defined as a vitamin D level less than 50 nmol/l ⁽²⁴⁾.

Despite the current appreciation of vitamin D's importance for child health and understanding that deficiency may cause not only rickets but also contribute to the magnitude of childhood illness and have serious implication for future life, there is lack of information on vitamin D status among pre-school children in developing countries ⁽¹⁶⁾. Hence, we aimed to assess the vitamin D status among children from 1 to 5 years old in rural Nepal, and to identify possible association between vitamin D status with socio-demographic indicators, breastfeeding practices and nutritional status in this population.

Methods

Study participants and setting

This community based cross- sectional study was conducted in rural Nepal at latitude 27.39° N during dry season between September and November 2012. Children from 1 to 5 years of age were recruited from the nine wards of Ugrachandi Nala Village Development Committee in Kavrepalanchowk district of Central Nepal. As a base for sampling we used records from Nepal's national vitamin A supplementation program for children aged 6 to 59 months with coverage of 90.4% as for 2011 ⁽²⁵⁾ available at local health post. Out of available records we created numbered list of all 411 children aged 12-60 months living at Ugrachandi Nala. By using online randomizer, a set of 320 unique sorted numbers was generated with range from 1 to 411 (representing the children assigned numbers). Subjects were selected randomly according to the produced list of numbers. Community Health Volunteers women were requested to identify selected children and invite guardians for participation in the study. We selected 320 children in order to achieve sample size of 280 subjects. Children with known metabolic bone disease, chronic disease associated with bone abnormalities or with current medication are likely to interfere with vitamin D metabolism were excluded from the study, as well as the children those guardians were not able to comply with study protocol and provide informed consent.

Ethical consideration

Present study was approved by the Nepal Health Research Council and Regional Committee for Medical and Health Research in Norway. Parents were explained about aims of the study and data collection procedure. Informed written or witness consent was obtained from the guardians of all participants. Witness consent was acquired when guardian had a decision making capacity but cannot read or write due to illiteracy. Witness was an adult from the same community who was not a member of study team.

Collection of Background information

Information regarding socio-demographic characteristic such as numbers of person in the household, parents' occupation, household income, as well as breastfeeding practices, milk product consumption and use of any vitamin supplementation, present and past child's health concerns and sun exposure was gained by trained study assistant through interview with guardians of the child by using structured questionnaire. The socio-economic status was assessed based on the household income and type of housing (made from natural or manufactured materials). The questionnaire was piloted among mothers at target setting. Pre-testing showed that children do not consume any food naturally rich with vitamin D. Consequently, question regarding vitamin D intake was removed from the questionnaire. Possible answers to the questions regarding to socio-demographic indicators were labelled based on the given responses with the option for unexpected answer. Milk product intake was calculated by multiplying the amount per serving and the frequencies of consumptions for each type of milk product. Calcium intake was computed by multiplying the calcium content of the specific type of milk by the daily consumed amount. Total calcium intake from milk products was calculated by summing the calcium intake from different milk products for each child. Calcium value in milk products were obtained from USDA National Nutrient Database for Standard Reference ⁽²⁶⁾. Weight was measured with digital weight scale made by Microlife Company in Swiss, certified for ISO 9001 accurate to 100g. Height was measured by wall stadiometer made by Bio-Plus 200cm, Model 265M/1013522. Anthropometric measurement were performed according to WHO Child Grow Assessment ⁽²⁷⁾.

Collection of blood and vitamin D analysis

Capillary blood was collected by a certified nurse after pricking the fingertip with a single use safety lancet. The total amount collected was approximately 0.5 ml. Blood drops were applied directly on the sampling filter card with pre-marked circles. The cards then were air dried during the first two hours; sample cards were stored in low gas –permeable zip lock bag with desiccant packages. Samples were kept at room temperature during the first 7 days, and later refrigerated. At the end of sample collection, vitamin DBS kits were shifted to the laboratory Vitas in Norway by DHL express for further analysis. The levels of 25(OH) D₃ and 25(OH) D₂ were quantified separately by using LC-MS/MS from DBS. Punches from DBS were added water, shaken, and diluted with 2-propanol/methanol containing the internal standard hexa-deuterio-25-hydroxy vitamin D₃ and BHT as an antioxidant. After mixing and centrifugation the supernatant was transferred to an insert, centrifuged again and an aliquot of 50µL was injected into the HPLC system. HPLC was performed with a Agilent 1200 liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA) interfaced by atmospheric pressure chemical ionization to a Agilent mass spectrometric detector operated in Multiple Reaction Monitoring mode. Vitamin D analogues were separated on a 4.6 mm x 150 mm reversed phase column with 2.7µm particles. The column temperature was 40 °C. A two-point calibration curve was made from analysis of DBS calibrators with known vitamin D concentrations. The inter-assay CV is 7.6 % (35.8 nM) and 10.2 % (78.3 nM).

The LC-MS/ MS DBS method was internally validated, it was chosen as a less invasive and convenient to use with paediatric groups in the field research. The laboratory performing the analysis is part of the Vitamin D Quality Assessment Scheme and compliant.

For the determination of vitamin D status there is not universally accepted threshold for normal level of 25(OH)D₃, however most experts agree that the lower limit of adequacy should be higher than 50nmol/l in order to avoid manifestation of rickets and osteomalacia ^(1, 3, 28). Although, there is a growing agreement that the optimal level should be between 75-100 nmol/l in order to maintain normal bone metabolism and ensure extra skeletal effects of vitamin D ⁽²⁹⁾. In this study we used reference range of 25(OH)D₃ suggested by Lips P., 2004 ⁽²⁸⁾, where vitamin D deficiency subdivided on mild, moderate and severe deficiency.

Assessment of nutritional status

The nutrition status of the children was determined by comparing measurements of weight for height, weight for age and height for age to the World Health Organization reference standards for wasting, underweight, and stunting indicators, respectively ⁽³⁰⁾.

1. weight-for-height (WFH) – wasting indicator (acute or recent malnutrition)
2. weight-for-age (WFA) – underweight indicator (acute or chronic malnutrition)
3. height-for-age (HFA) – stunting indicator (chronic or long-standing malnutrition)

Also global acute malnutrition (wasting) is defined as <-2 z scores weight-for-height and/or oedema. Severe acute malnutrition is defined as <-3 z scores weight-for-height and/or oedema. Underweight is defined as <-2 z scores weight-for-age while severe underweight is defined as <-3 z scores weight-for-age. Chronic malnutrition (stunting) is defined as <-2 z scores height-for-age while severe chronic malnutrition is defined as <-3 z scores height-for-age.

Statistical analysis

Statistical analysis of the data was performed by using PASW software (version 20, SPSS IBM Corporation, USA). WHO Anthro software (version 3.2.2), was used to evaluate gender specific growth indicators in relation to WHO standards ⁽³⁰⁾. Standing height for children younger than 24 months was equalized to the recumbent length by adding 0.7 cm. according to WHO Anthro software's guidelines. Data from descriptive statistic are presented as mean scores and standard deviation. To compare the mean scores of vitamin D level between categorical variables we used independent sample t-test (two-tailed). One way analysis of variance was performed to explore association between vitamin D concentrations with the categorical explanatory variables. For unequal variances the non-parametric test has been used (Kruskal- Wallis test, Mann-Whitney U test). Results have been considered statistically significant with p value below 0.05.

Results

Most of the children (81.1%) from our sample (n=280; 157 boys and 123 girls) were from low to medium socio-economic group. The majority (95%) of the children belonged to Hindu families. The basic characteristics of children are given in Table 1. The vast majority of parents reported trousers and long sleeves shirt as regular clothing for their children; 16% mentioned t-shirt as an option for warm days. In our sample breastfeeding was almost universal (99.3%), with 107 mothers reported that child is still breastfeed. The mean duration for exclusive breastfeeding was estimated 6 months while the mean duration for breastfeeding in general was estimated at 27 months. Over half of the children in our sample were stunted, while nearly one-fourth were underweight.

Table 1, Basic characteristics of children (mean value, standard deviation, number of subjects and percentages)

	Girls (n= 123)		Boys (n= 157)	
	n (%)	mean (SD)	n (%)	mean (SD)
Age (months)				
12-24	42 (34.1)	—	51 (32.5)	—
25-36	35 (28.5)	—	37 (23.6)	—
37-60	46 (37.4)	—	69 (43.9)	—
Weight (kg)	—	11.5 (2.5)	—	12.3 (2.6)
Height (cm)	—	86.6 (10.2)	—	89.1 (9.9)
Calcium intake (mg/day)	—	289 (203)	—	340 (256)
Currently breastfeed	47 (38.2)	—	60 (38.2)	—
25(OH)D₃ (nmol/l)	—	31.2 (11.2)	—	31.9 (12.6)
Weight-for –height				
-Global acute malnutrition	4 (3.4)	—	8 (5.4)	—
-Severe acute malnutrition	1 (0.8)	—	2 (1.4)	—
Weight-for-age				
-Underweight	27 (22.0)	—	20 (12.7)	—
-Severe underweight	8 (6.5)	—	11 (7)	—
Height-for-age				
-Chronic malnutrition	37 (30.8)	—	37 (23.6)	—
-Severe chronic malnutrition	21 (17.5)	—	24 (16.3)	—

Vitamin D status

Concentration of 25(OH)D₃ ranged from 6.9 to 74.5 nmol/l with a mean score of 31.6nmol/l (SD 12.0); Around 91.1% of the children had levels of 25(OH)D₃ less than 50 nmol/l. Table 2 classifies states of vitamin D deficiency on the basis of 25(OH)D₃ levels. As related to the level of 25(OH)D₂ for the 96. 8% of children it was not detectable (less than 5nmol/l).

Table 2, Vitamin D status in relation to 25(OH)D₃- levels among 1-5 year old children in rural Nepal

Vitamin D status	Cut-offs	Prevalence among study population
		% (n)
Sufficient	≥ 50 nmol/l	8.9 (25)
Mild deficiency	25.1-49.9 nmol/l	61.8 (173)
Moderate deficiency	12.6-25 nmol/l	25.4 (71)
Sever deficiency	0-12.5 nmol/l	3.9 (11)

There was no difference in the mean 25(OH)D₃ levels between boys and girls. The mean 25(OH)D₃ levels in those children younger than 36 months who were currently breastfeed (36.4 nmol/l, SD 13.2) were higher compare to those who were not (28.6 nmol/l, $P=0.001$). Younger children had mean level of 25(OH)D₃ higher in comparison to the older group ($P=0.01$). While other variables such as socio-economic indicator, measured as a monthly household income, gender, sun exposure and nutritional status were not associated with levels of 25(OH)D₃

Table3, Characteristics of study population by 25(OH)D₃ level

Characteristic	N	(%)	Mean 25(OH)D ₃ level nmol/l	(SD)	p- Value [*]
Gender					
Female	123	(43.9)	31.2	(11.2)	0.6
Male	157	(56.1)	31.9	(12.6)	
Age(months)					
12-24	93	(33.2)	35.4	(13.1)	0.01
25-36	72	(25.7)	30.8	(11.4)	
37-60	115	(41.1)	29.0	(10.7)	
Household income (monthly, NRS ^{**})					
Below 14999	140	(50)	32.7	(12.2)	0.2
15000-39999	87	(31.1)	31.1	(11.7)	
Above 40000	53	(18.9)	29.4	(11.9)	
Currently breastfeed (for children 12-36m)					
Yes	102	(61.8)	36.4	(13.2)	0.001
No	63	(38.2)	28.6	(9.8)	
Nutritional status					
Weight-for height					
-Corresponds to normal	251	(94.4)	31.2	(11.6)	0.11
-Global acute malnutrition ^{***}	15	(5.6)	38.2	(15.7)	
Weight-for-age					
-Corresponds to normal	214	(76.4)	31.1	(11.7)	0.98
-Underweight	47	(16.8)	31.6	(11.4)	
-Severe underweight	19	(6.8)	37.5	(15.4)	
Height-for-age					
-Corresponds to normal	148	(55.4)	31.7	(12.2)	0.74
-Chronic malnutrition	74	(27.7)	31.04	(11.8)	
-Severe chronic malnutrition	45	(16.9)	32.8	(11.6)	

* p -value was calculated by using T-test, ANOVA and Kruskal-Wallis test for non-normally distributed variances

** NRS: Nepalese Rupees. 1USD = approx. NRS 100 as of September 30, 2012.

*** Nutritional status determined with cut-offs -2SD, includes 3 cases with score below -3SD

Discussion

We found that vitamin D deficiency is common among children in rural Nepal, with up to 91.1% of children between 1-5 years of age having a 25(OH)D₃ level under 50nmol/L. The findings of the current study are consistent with the few reports of vitamin D status among neonates, adolescent and adults in the region ^(20, 22, 31-33). Similar prevalence of vitamin D deficiency was identified in case-controls studies conducted among children of preschool age in India and Bangladesh. Wayse et al. using the same cut off for the determination of vitamin D status recorded vitamin D deficiency 95% and 61% for cases and controls respectively ⁽³⁴⁾. Similarly Roth et al. indicated mean serum 25-hydroxyvitamin D concentration 29.2nmol/l for cases and 39.2nmol/l for controls ⁽³⁵⁾. Calcium intake from milk products was recorded as uniformly low 318.08 Ca mg/day (SD 235.4), it also was observed by field staff, children get quite often cow or buffalo milk and homemade yogurt but the quantities per serving is very small.

Factors associated with vitamin D status

In contrast to earlier findings, we did not find significant difference between vitamin D level's in boys and in girls, as it was observed by Sahu et al. in rural India for teenagers ⁽³⁶⁾, it could be explained that in this age group there is no difference in clothing practices and outdoor activities between genders. In our sample we did not observe difference in vitamin D levels of children between different social-economic layers, measured as income level and type of housings, as it was for Pakistani infants ⁽³⁷⁾ and Indian schoolgirls ⁽³²⁾. It might not be

appropriate to mention that discrepancy as a contradiction given the fact that the study population differ in age. High level of vitamin D among low socioeconomic strata explained with prolonged sun exposure due to occupational particularities, inversely better household condition and education leads to restriction of outdoor activities ^(37, 38). As related to our study population, it seems that for children in preschool age vitamin D status is not determined with socio-economic conditions because of similarly poor vitamin D supply in diet and comparable exposure to sunlight.

In the literature exclusive long-lasting breastfeeding was reported as a risk factor for the development of hypovitaminosis D ^(1, 39). In our study we were not able to test that association, taking into consideration that our subjects were older than one year of old, and were not breastfeed exclusively. Although, we found that children who were received breast milk in addition to traditional food had better vitamin D status compare to those who were not. Also younger children had significantly higher levels of vitamin D than those in the older groups. Contrary to our expectations, we did not find a significant difference in the mean scores of 25(OH)D₃ for the children with different nutritional status.

Even though this finding is in agreement with similar studies conducted in the region, the reason for this high magnitude of not adequate vitamin D status remains unclear. Several explanations have been postulated. These include intensive skin pigmentation, traditional diet poor with natural source of vitamin D, long lasting exclusively breastfeeding of infants and absents of national vitamin D supplementation program ^(15, 40).

Limitation

This study has several limitations. For the drawing sample from population the randomization was applied. Although, due to the absence of demographic data in the region, we have had to create our own databases from the vitamin A supplementation program, records available at VDC health post. It means that, children that for some reasons did not get vitamin A supplements during last 6 months were not included in our databases, and consequently did not get chance to be selected for the current study. This kind of selection bias may result that our sample were more representative for those who has better access to health care programs than for general population in total. Apparently calcium intake was not calculated accurately, we used reported regular consumption of milk products, but we accept that it might be recalled inaccurately, also, we did not count other sources of calcium in the diet.

Question regarding sun exposure was not enough detailed, it did not clarify time of being outside during the day. It is important due to the effect on the vitamin D production in the skin under the impact of UVB. Another aspect that affects the quality of data is approximation in the age of children. Nepalese people do not register children immediately after birth. Situation with clarifying an age was aggravated because of differences in calendar year, between lunisolar Hindu calendar officially used in Nepal and western Gregorian one. So, we registered the age of the child based on the mother's claiming, but we accept deviations of 6-12 months. Yet another limitation is that we, for weight and height measurements used equipment that were not standardised for establishing of anthropometric databases. It might be considered as a limitation the absence of data on the level of calcium, phosphorus and alkaline phosphatase from our study population. Although, these indicators are more relevant to the assessment of bone metabolism and deviate during severe vitamin D deficiency. In fact, simple measurement of 25-hydroxyvitamin D₃ reflects the objectives of our study to assess the vitamin D status of children.

Despite of these limitations this study shapes general pattern of vitamin D status among pre-school children in rural Nepal. It was designed with adequate power and sufficient sample size for the first time among pre-school children in the region. Full sample size was achieved with respond rate of 87.5%, vitamin D status was assessed with DBS method internally validated. However we should take into consideration that Nepal is country with tremendous diversity in altitude, which has effect on the vitamin D production through the whole year⁽⁴¹⁾. Therefore this result needs to be interpreted with respect to study population and geographical coordinates.

Conclusion

We report high prevalence of vitamin D deficiency among pre-school children in rural area of Nepal at latitude 27.39° N. In our sample, socio-demographic factors did not affect on vitamin D status of children, but prolonged breastfeeding practices in late infancy was associated with better vitamin D level. Further research is required to investigate the health consequences of low vitamin D status for this population.

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